# Saethre-Chotzen mutations cause TWIST protein degradation or impaired nuclear location

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# SAETHRE-CHOTZEN MUTATIONS CAUSE TWIST PROTEIN DEGRADATION OR IMPAIRED NUCLEAR LOCATION

Vincent El Ghouzzi, Laurence Legeai-Mallet, Sandra Aresta[1], Catherine Benoist, Arnold Munnich, Jean de Gunzburg[1] and Jacky Bonaventure[+].

Unité de Recherches sur les Handicaps Génétiques de l'Enfant. INSERM U-393, Institut Necker, 149, rue de Sèvres, Paris, France, [1]INSERM U-248, Institut Curie. Section de Recherche, 26, rue d'Ulm , Paris, France

[+]To whom correspondence should be addressed. Tel: +33 1 44 49 40 00 ext.97833; Fax: +33 1 47 34 85 14; Email: bonavent@necker.fr

**ABSTRACT**

**H-TWIST belongs to the family of basic helix-loop-helix (bHLH) transcription factors known to exert their activity through dimer formation. We have recently demonstrated that mutations in H-TWIST account for Saethre-Chotzen syndrome (SCS), an autosomal dominant craniosynostosis syndrome characterized by premature fusion of coronal sutures and limb abnormalities of variable severity. Although insertions, deletions, nonsense and missense mutations have been identified, no genotype-phenotype correlation could be found, suggesting that the gene alterations lead to a loss of protein function irrespective of the mutation. To assess this hypothesis, we studied stability, dimerization capacities and subcellular distribution of three types of TWIST mutants. Here, we show that *i)* nonsense mutations resulted in truncated protein instability, *ii)* missense mutations involving the helical domains led to a complete loss of H-TWIST heterodimerization with the E12 bHLH protein in the two-hybrid system and dramatically altered the ability of the TWIST protein to localize in the nucleus of Cos-transfected cells, *iii)* in-frame insertion or missense mutations within the loop significantly altered dimer formation but not the nuclear location of the protein. We conclude that at least two distinct mechanisms account for loss of TWIST protein function in SCS patients, namely protein degradation and subcellular mislocalization.**

## INTRODUCTION

Saethre-Chotzen syndrome is an autosomal dominant craniosynostosis characterized by premature fusion of the coronal sutures leading to skull deformation, distal limb abnormalities and dysmorphic facial features of variable severity. This disorder has been ascribed to mutations in the *H-Twist* gene encoding a basic helix-loop-helix (bHLH) transcription factor (1,2). *Twist* has been first identified in *Drosophila* as a zygotic developmental gene involved in early mesoderm patterning (3,4). By contrast, its murine homologue *M-Twist* is not critical for mesoderm formation as homozygous *Twist*-null embryos die at embryonic day 11.5 after mesoderm has formed (5). The specific expression of *M-Twist* transcripts in cephalic and branchial mesectoderm and in subsets of mesodermal cells (6,7) indicates that TWIST belongs to the class B bHLH proteins. Most of these transcriptional factors are known to form stable heterodimers with ubiquitous class A bHLH proteins including members of the E family (8,9) although variations among species have been reported. Indeed, Ce-TWIST, the *Caenorhabditis elegans* homologue of H-TWIST, heterodimerizes with the E/daughterless homologue to activate target genes (10). By contrast the *Drosophila* D-TWIST protein is able to form homodimers and does not appear to require heterodimerization for DNA binding (11). In the mouse model, M-TWIST has been shown to form either homodimers or heterodimers *in vitro* (12,13). Whether H-TWIST forms homodimers or heterodimers in human, therefore remains to be elucidated.

The identification of single base mutations or deletions producing premature termination codon in the *H-Twist* gene and the SCS-like phenotype of *Twist*-null heterozygous mice strongly suggest that haploinsufficiency is the pathogenic mechanism underlying SCS (1,2,14,15). That gene alterations could result in a loss of protein function irrespective of the mutation is further supported by the absence of genotype-phenotype correlation among patients carrying nonsense, missense or in-frame insertions. However, the hypothesis has been raised that some missense mutations could confer a dominant negative function to the protein (16). In an attempt to understand how mutations affected the TWIST protein, cDNAs carrying different types of mutations identified in SCS patients

were generated and used for *in vitro* and *in vivo* experiments. Here we show by transfection assay and immunoblotting that nonsense mutations result in the synthesis of truncated proteins that are rapidly degraded whereas other mutant proteins are stable. By using a two-hybrid assay, we demonstrate that the human E12 protein is a partner for heterodimerization with H-TWIST. Dimer formation is abolished by missense mutations in the TWIST helical domains but not by in frame insertion or missense mutations in the loop. This helix/loop correlation is further supported by immunostaining experiments showing that unlike mutations in the loop, helix-mutations dramatically reduce the number of TWIST nuclear positive cells.

## RESULTS

### Nonsense mutations induce degradation of truncated TWIST proteins

To determine how *H-Twist* mutations alter protein function in SCS, human *Twist* cDNAs carrying different types of mutations including three nonsense mutations (Y103X, E126X, Q161X), three missense mutations affecting either the helices (A129P, helix I; L159F, helix II) or the loop (K145E) of the HLH motif and a 21bp in-frame insertion (P139ins7) were generated (Fig. 1a). *Twist* mutants cloned in an expression vector were *in vitro*-translated in a reticulocyte lysate system and resulted in molecules of the expected size (Fig. 1b). RNA and protein stability was then tested by ectopically expressing wild-type and mutant constructs in transfected Cos7 cells. Northern blot analysis of total RNAs detected similar amounts of *Twist* transcripts irrespective of the mutation, indicating that RNA stability was unaffected (not shown). Immunoblot analyses of cell lysates revealed that truncated proteins due to nonsense mutations were unstable as their expression level was reduced 24h post-transfection as compared to the wild-type (Fig. 1c) and were undetectable after 48h (Fig. 1d). By contrast, the amount of missense and insertion mutant gene products was similar to control (Fig. 1c,d). The variable stability of mutant proteins suggested that markedly different mechanisms should cause the SCS phenotype.

**Direct interaction between H-TWIST and E12 *in vitro* and *in vivo***

Since bHLH transcription factors require either homodimerization or heterodimerization for DNA binding, we tested by using a two-hybrid assay whether H-TWIST could form homodimers or heterodimers. The entire coding sequence of the gene was expressed in yeast as fusions with either the LexA DNA-binding domain or the GAL4 activation domain (GAD) and both constructs were co-transformed in yeast. Co-expression of wild-type LexA-TWIST and GAD-TWIST fusion proteins failed to transactivate HIS3, as evidenced by their inability to grow on medium lacking histidine (Fig. 2a). By contrast, co-expression of wild-type LexA-TWIST and GAD-E12 fusion proteins led to rapid yeast growth on medium lacking histidine with the same efficiency as LexA-rap2A/GAD ral-GDS (17) used as a positive control (Fig. 2a). Similar results were obtained with a β-galactosidase reporter gene (not shown). We conclude that H-TWIST does not homodimerize but can efficiently interact with the human E12 protein in the yeast two-hybrid system. Additional evidence for this interaction was derived from binding assays of *in vitro*-translated wild-type and mutant proteins to the glutathione S-transferase-E12 fusion protein (GST-E12). Indeed, H-TWIST directly interacted with E12 fused to GST and deletion of the bHLH domain from either partners hampered their interaction, thus indicating that these domains are required for heterodimerization (Fig. 2d). Finally, H-TWIST and E12 also interacted in mammalian cells co-transfected with expression constructs, as evidenced by co-immunoprecipitation of the two partners (Fig. 2e).

**Analysis of TWIST mutant proteins by the two-hybrid assay**

We next investigated whether the TWIST/E12 interaction could be altered by TWIST mutations. Mutant constructs fused to the LexA DNA binding domain were co-expressed in yeast with a GAD-E12 fusion protein and their capacity to interact was assessed as described above. Interestingly, two distinct patterns of yeast growth were observed (Fig. 2b). First, nonsense mutations consistently led to a complete loss of interaction with E12, irrespective of the extent of protein truncation. Similarly, the two missense mutations affecting either helix of the HLH motif failed to transactivate the

reporter gene when co-expressed with E12. The absence of transactivation of the HIS3 gene could not be ascribed to the lack of expression of the mutant protein, as evidenced by immunoblotting with an anti-TWIST antibody (not shown). Second, missense mutations and the in-frame insertion within the loop were still able to lead to histidine auxotrophy (Fig. 2b) albeit quantitation of β-galactosidase activity revealed a 10 to 50-fold decreased interaction as compared to the wild-type heterodimer (Fig. 2c). Hence, protein truncation and mutations of the helix domains hampered the ability of TWIST to interact with its partner while proteins carrying loop mutations were still able to form dimers with E12.

**Subcellular localization of the TWIST mutant proteins**

The M-TWIST protein has been detected in the cell nuclei of normal mouse embryos (18). Considering that the subcellular localization of transcription factors in either the cytoplasm or the nucleus regulates their biological activity, we wondered whether an abnormal subcellular distribution of the mutant H-TWIST protein could account for the disease. To address this issue, TWIST mutant proteins carrying either the in-frame insertion or missense mutations were co-expressed with E12 in Cos7 cells and the localization of both proteins was examined by immunofluorescence microscopy. Cells transfected with wild-type constructs exhibited a strong nuclear staining with either anti-TWIST or anti-E12 antibodies (Fig. 3a-c) while no signal was detected in untransfected cells (Fig. 3p-r). Three patterns of immunofluorescence were observed using the transfected mutant constructs: *i)* mutations altering helices caused a marked staining of the cytoplasm using anti-TWIST antibodies but these mutations had no effect on the localization of E12 which remained in the nucleus (Fig. 3d-i); *ii)* missense mutations within the loop did not alter the nuclear localization of the TWIST mutant protein (Fig. 3m-o); *iii)* the in-frame insertion gave an intermediate figure, as shown by the presence of cytoplasm-positive and nuclear-positive cells (Fig. 3j-l).

These observations were further documented by quantifying the proportion of cells showing either a nuclear or a cytoplasmic localization of TWIST. While more than 90% of cells expressing

the wild-type protein or the loop mutant exhibited a normal nuclear staining, this percentage fell to 25-40% for mutations altering TWIST helices (Fig. 4a). These data were further confirmed by fractionating transfected cells into nuclear and non-nuclear fractions. Immunoblotting revealed that the wild-type protein and the loop mutant were only detectable in the nuclear fraction. Helix mutants were mostly present in the cytoplasm while mutant proteins carrying the insertion were detected in both fractions (Fig. 4b). However, in both assays the L159F helix mutant was apparently more cytoplasmic than the A129P helix mutant.

## DISCUSSION

Although compelling evidence now exists that different types of mutations in the *Twist* gene account for common clinical features including craniofacial and limb anomalies, the phenotypic variability seen in SCS patients and the absence of genotype-phenotype correlation is far to be understood (14,19). The present study was thus undertaken to determine how mutations in the *H-Twist* gene affected the protein function. To this purpose, a series of recombinant plasmids carrying mutations identified in SCS patients were produced. Expression analyses first demonstrated that nonsense mutations resulted in the synthesis of truncated proteins that were rapidly degraded, thereby leading to haploinsufficiency. Along these lines, it is worth keeping in mind that individuals carrying monosomy 7p21 and thus hemizygous for the *Twist* gene had a SCS phenotype (20). Similarly, *Twist*-null heterozygous mouse model mimicked the human phenotype (1,15).

Since missense mutations or in frame insertion had no detectable effect on the stability of the translation product in transfected cells, we speculated that these mutations altered the TWIST function through a different mechanism. Class B bHLH proteins to which H-TWIST belongs are known to form stable heterodimers with members of class A bHLH transcription factors including gene products of E2A (E12 and E47) (8,9), E2-2 and E2-5 (ITF-2 and ITF-1 respectively) (21) or HEB/HTF-4 (22). However, D-TWIST homodimerization has been reported in *Drosophila* (11) and homodimerization of the mouse M-TWIST protein has been observed in an *in vitro* binding assay

(13). The yeast two-hybrid assay thus appeared as a relevant model to test the possible H-TWIST homodimerization or heterodimerization with a member of the E protein family (23). We found no homodimerization of the H-TWIST protein in this *in vivo* system further emphasizing the striking differences between *in vitro* and *in vivo* HLH dimerization (24). By contrast, E12 strongly interacted with H-TWIST in yeast. This finding was consistent with the previous observation that E12 was able to heterodimerize with M-TWIST in an *in vitro* assay (12). In addition, DERMO-1, a bHLH transcription factor closely related to M-TWIST, has emerged from a two-hybrid screen for factors interacting with E12 (25). The capacity of H-TWIST to form stable heterodimers with E12 was further supported by *in vitro* binding experiments and co-immunoprecipitation studies. Hence, E12 appears as a putative partner *in vivo* for regulating the H-TWIST transcriptional activity. Interestingly, *Twist* and *E2A* transcripts have been detected in mouse osteoblastic cells (26) and were found to be co-expressed in the developing mandible and limb buds of human embryos (unpublished data). Based on the recent demonstration that H-TWIST has an inhibitory effect on osteogenic differentiation (27), the TWIST/E12 heterodimer might be regarded as a negative regulator of transcription in human osteoblastic cells.

Heterodimerization proved to be mediated through the bHLH motif as deletion of the E12 bHLH domain or nonsense mutations truncating the TWIST bHLH domain hampered heterodimer formation. This result is in accordance with previous *in vitro* data obtained with MyoD (28), E47 (29), Lyl-1 (30) but does not rule out the possible influence of additional adjacent HLH sequences for *in vivo* dimerization (24). Within the HLH motif, the two helices seem to play a more critical role than the loop as their alteration results in both a markedly impaired heterodimerization and an abnormal cellular location of the protein. Likewise, the helix 2 mutation affecting a highly conserved leucine residue at position 159 had a more dramatic effect on the protein mis-localization than the A129P substitution in helix 1. This observation is consistent with the critical role of leucine residues in $\alpha$ helices stability (8). Interestingly, significant increase of the loop length due to in-frame insertion of seven amino-acids had little effect on dimer formation and nuclear location. Similarly, random mutations within the loop region of E47 do not alter protein dimerization (29)

and replacement of five residues of the loop in M-TWIST fail to abolish its interaction with MyoD or E12 (13).

Mutations in transcription factors may affect their nuclear localization and thus account for their inability to bind DNA (31). Accordingly, the subcellular localization of mutant TWIST proteins was investigated in Cos cells following co-transfection with E12. Since heterodimerization of HLH proteins has been proposed to take place in the cytoplasm prior to nuclear import (32), the expected nuclear location of the wild-type TWIST protein appears to be a direct reflection of the *in vivo* interaction with E12. Consequently, the cytoplasmic location of TWIST proteins carrying single amino-acid changes in the helices might result from the loss of dimer formation. Alternatively, a rapid nuclear export of the mutant protein could account for its abnormal subcellular distribution (33). Nevertheless, the normal nuclear location of loop mutants suggests that loss of TWIST function in these cases occurs at still another step. Alteration of the DNA binding properties of the TWIST/E12 complex is the most likely explanation but will require identification of human target DNA sequences to be confirmed.

## MATERIALS AND METHODS

### cDNAs constructs and expression vectors.

For eukaryotic expression in Cos7 cells and *in vitro* expression of TWIST proteins, genomic DNA from control and SCS patients was PCR amplified and amplification products were directly cloned into the pCR3 unidirectional plasmid (Invitrogen, San Diego CA). Primers used for PCR allowed generation of a 5'*BamHI* and a 3'*SalI* restriction site used for subsequent subcloning. Normal and mutant inserts were sequenced to ensure integrity of the cloned allele. For E12 expression, a human full-length cDNA in a pSP65 vector was subcloned into the pCMV5 vector. For expression of the LexA fusion protein in yeast, we used the pVJL10 plasmid (34). Wild-type and mutant Twist cDNAs were subcloned from pCR3uni into pVJL10 using the *BamHI* and *SalI* restriction sites. The fusion between the activation domain of GAL4 and TWIST was generated by subcloning the wild type Twist cDNA from pCR3uni into pGAD1318 using *BamHI* as the 5' restriction site and *SalI/XhoI* half sites in 3'. The GAL4 activation domain-E12 fusion was obtained by subcloning the E12 cDNA into the pGAD-GE plasmid using the *EcoRI* site. To produce GST-E12 fusion proteins, the full-length and a bHLH-deleted form of E12 were subcloned into the pGEX4T-1 vector (Pharmacia) between the *EcoRI* and *XhoI* sites.

### Cell cultures, transfection and immunofluorescence.

Cos7 cells were grown in DMEM supplemented with 10% Fetal Calf Serum (FCS) and antibiotics. Six-well tissue culture plates and 8-well chamber slides were seeded with 1.5 X $10^5$ and $10^4$ cells/well respectively and cultured to 70% confluency. Transfection were performed with Fugene-6 (Roche) and 2μg or 0.1μg DNA/well in FCS supplemented DMEM according to the manufacturer instructions. Twenty four or 48h later, cells were used for immunofluorescence studies. Transfected cells in culture chamber slides were fixed with 4% paraformaldehyde for 30 min. After permeabilization with 0.1% Triton X-100 for 15 min, cells were incubated for 1h at room temperature with the above mentioned primary antibodies at the respective dilutions of 1:100 and

1:1000. Cells were then incubated with either a Fluorescent Isothiocyanate (FITC)-conjugated donkey anti-goat antibody (Santa Cruz Biotechnology) to visualize TWIST or a Cy3-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Lab) to visualize E12. Cells were examined with a Zeiss LSM 510 confocal microscope.

**Immunoblotting and co-immunoprecipitation.**

Cell lysates were denatured by boiling in sample buffer, separated by 7% or 12% SDS-PAGE and electroblotted on PVDF membranes (Immobilon, Millipore Corp). The membranes were pre-incubated for 1h at 4°C in TBS (20 mM Tris-HCl pH 7.4, 150 mM NaCl) containing 5% skim milk. The blots were then incubated with either goat polyclonal anti-TWIST or mouse monoclonal anti-E12 antibodies (Santa Cruz Biotechnology) at respective dilutions of 1:200 and 1:100 overnight at 4°C. After membrane washing, a second antibody coupled to peroxydase was added. Proteins were visualized with the ECL detection kit (Amersham). Co-immunoprecipitation was achieved by adding 5µg of anti-E12 antibody to 50µl of cell lysate followed by incubation at 4°C for 2h. Protein A-sepharose beads were added and the mixture was rotated at 4°C for 1h. Beads were washed twice with 0.1M Tris-HCl pH7.6 buffer containing 0.25M NaCl, and twice with 10mM Tris-HCl pH7.6, 0.1M NaCl, 1mM EDTA then resuspended in 40 µl of SDS-PAGE sample buffer and boiled for 5 min. Cytoplasmic and nuclear extracts were prepared as described (35). Both fractions were submitted to immunoblot analysis as described above.

**Yeast two-hybrid assay**

The genotype of the *Saccharomyces cerevisiae* reporter strain L40 was MATa *trp1 leu 2 his3 ade2*, *LYS2::lexA-HIS3 URA3::lexA-lacZ*. Yeast strains were grown at 30°C in a rich medium (1% yeast extract, 2% Bacto-peptone, 2% glucose) or in a synthetic minimal medium containing appropriate supplements. The yeast reporter strain L40, which contains the reporter genes *LacZ* and *HIS3* downstream of the binding sequences for LexA, was co-transformed with pVJL10-TWIST (wild-type or mutants) and either pGAD1318-TWIST (wild-type) or pGAD-GE-E12 plasmids using the

lithium acetate method. Double transformants were plated to synthetic medium lacking leucine and tryptophan. The plates were incubated at 30°C for 2 days. $Leu^+Trp^+$ colonies were patched on selective plates lacking histidine and assayed for β-galactosidase activity by a filter assay (36). Quantification of β-galactosidase activity was performed as described (37).

### *In vitro* protein-protein interaction assay

Wild-type and mutant human Twist cDNAs were transcribed and translated *in vitro* from pCR3uni constructs using the TNT T7 Quick transcription/translation system (Promega,) in the presence of $^{35}$S-Methionine (1000 Ci/mmol, Amersham). Synthesis of radio-labelled proteins was checked by SDS-PAGE in a 12% polyacrylamide gel, followed by fluorography. BL21 *E.Coli* cells, transformed with pGEX4T-1/$E12_{wt}$ or $E12_{\Delta bHLH}$ were incubated for 3h at 30°C in the presence of 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) to induce the production of wild-type GST-E12 and bHLH-deleted GST-E12 fusion proteins respectively. Recombinant GST fusion proteins were purified on glutathione-Sepharose 4B beads (Pharmacia). Purity and yield of the GST-fusion proteins were assessed by SDS-PAGE in 7% polyacrylamide gels stained with Coomassie Blue.

*In vitro* interaction assays were performed by incubating 10µl GST-E12 beads with 5µl $^{35}$S-TWIST at 4°C for 1h in a 50mM Tris HCl pH 7.5, 100mM NaCl, 5mM $MgCl_2$ buffer containing a mixture of protease inhibitors (Roche) and 0.1% X-100 Triton. Beads were washed six times for 2min in the same buffer containing 125mM NaCl and 1% X-100 Triton, boiled in SDS-PAGE sample buffer and analyzed in 12% polyacrylamide gels.

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## LEGENDS TO FIGURES

### Figure 1.

Instability of truncated TWIST proteins. (**a**) Diagram of wild-type and mutant TWIST proteins reproducing seven SCS mutations. The helices (H), the basic (b) and the loop (L) regions of the bHLH domain are represented by white, gray and black boxes respectively. The positions of the in-frame insertion and missense mutations are shown by a horizontal bar and black diamonds respectively. (**b**) Gel electrophoresis analysis of *in vitro*-translated wild-type and mutant proteins labeled with $^{35}$S-methionine. (**c**) Immunoblot analysis of the H-TWIST protein in Cos7 cells transfected with wild-type (lane 1) and mutant expression vectors carrying nonsense mutations (lanes 2,3,4), missense mutations (lanes 5,6,7), or a 21bp insertion 24h post-transfection (lane 8). (**d**) Immunoblot analysis of the same mutants 48h post-transfection. Similar amounts of protein (15µg) were loaded in each lane as shown by a mouse monoclonal anti β-actin antibody (lower panels).

### Figure 2.

Identification of E12 as a dimerization partner for TWIST and analysis of Twist mutants by using the yeast two-hybrid system. (**a**) Two-hybrid assay showing the absence of TWIST homodimerization (lane 4) and strong heterodimerization with E12 (lane 6). Rap2B/RalGDS interaction was used as positive control (lane 7). Yeast expressing LexA and GAD alone were used as negative control (lane 1). Co-transformed yeast were selected on a $leu^-$, $trp^-$ medium (lower panel) and protein-protein interaction was visualized by testing the ability of yeast to grow on a $leu^-$, $trp^-$, $his^-$ medium (upper panel). (**b**) Truncated TWIST proteins failed to heterodimerize (lanes 1-3); the L159F and the A129P mutants disclosed no interaction (lanes 4, 5); the P139ins7 and K145E mutants still heterodimerize with E12 (lanes 6, 7). (**c**) Histogram of β-galactosidase activity to measure interaction between E12 and either wild-type or mutants. (**d**) *In vitro* interaction between

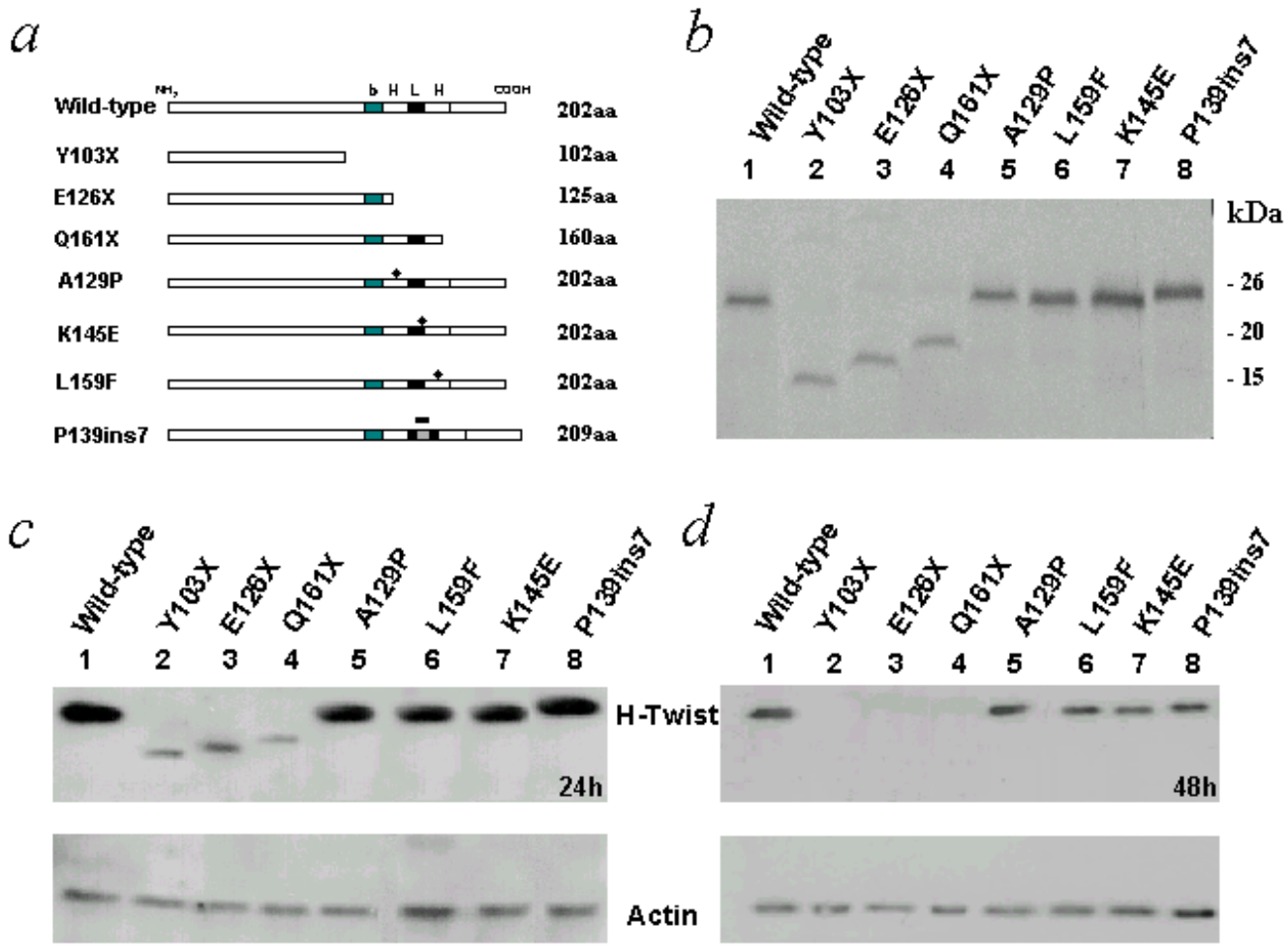
$^{35}$S-labelled TWIST and GST-E12. Interaction between the two wild-type proteins (lane 1) was lost upon deletion of the bHLH domains of either TWIST (lane 2) or E12 (lane 3). No interaction occured between GST alone and TWIST (lane 4). The amount of radio-labelled TWIST protein used for the assay is shown as an input (lane 5). (**e**) Co-immunoprecipitation of the TWIST/E12 complex. Lysates from cells co-transfected with expression vectors encoding wild type TWIST and E12 proteins were immunoprecipitated with an anti-E12 antibody, separated on 12% SDS-PAGE and immunoblotted with the same antibody (lanes 1,2) or an anti-TWIST antibody (lanes 3,4). Immunoglobulin heavy and light chains are indicated by H and L.

**Figure 3.**

Subcellular immuno-localization of the wild-type and mutant TWIST proteins in Cos7 cells co-transfected with E12. (**a**-**c**) Wild-type TWIST and E12 proteins were co-localized in the nucleus. (**d-i**) The A129P and L159F TWIST mutants disclosed a predominant cytoplasmic localization whereas E12 remained in the nucleus. (**j**-**l**) The 21bp in-frame insertion mutant (P139ins7) was detected in both the cytoplasmic and the nuclear compartments. (**m**-**o**) The K145E mutant gave the same pattern as the wild-type. (**p** and **q**) Negative controls were obtained by incubating cells with either the FITC-conjugated anti-goat antibody or the Cy3$^{TM}$-conjugated anti-mouse antibody. (**r**) Phase contrast view of Cos 7 cells, bar = 20µm.

**Figure 4.**

Sub-cellular distribution of the wild-type and mutant TWIST proteins in Cos7 cells co-transfected with E12. (**a**) Histogram representing the proportion of FITC-nuclear positive transfected cells pending on the mutation. At least 200 cells were counted by three independent investigators. Results shown are the mean +/- S.D. of three separate experiments. (**b**) Immunoblot analyses of cytoplasmic (left) and nuclear fractions (right) isolated from co-transfected cells. The blots were sequentially incubated with anti-TWIST and anti-E12 antibodies.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NH2
b H L H
COOH
Wild-type
202aa
Y103X
102aa
E126X
125aa
Q161X
160aa
A129P
202aa
K145E
202aa
L159F
202aa
P139ins7
209aa
b
Wild-type
Y103X
E126X
Q161X
A129P
L159F
K145E
P139ins7
1 2 3 4 5 6 7 8
kDa
- 26
- 20
- 15
c
Wild-type
Y103X
E126X
Q161X
A129P
L159F
K145E
P139ins7
1 2 3 4 5 6 7 8
H-Twist
24h
Actin
d
Wild-type
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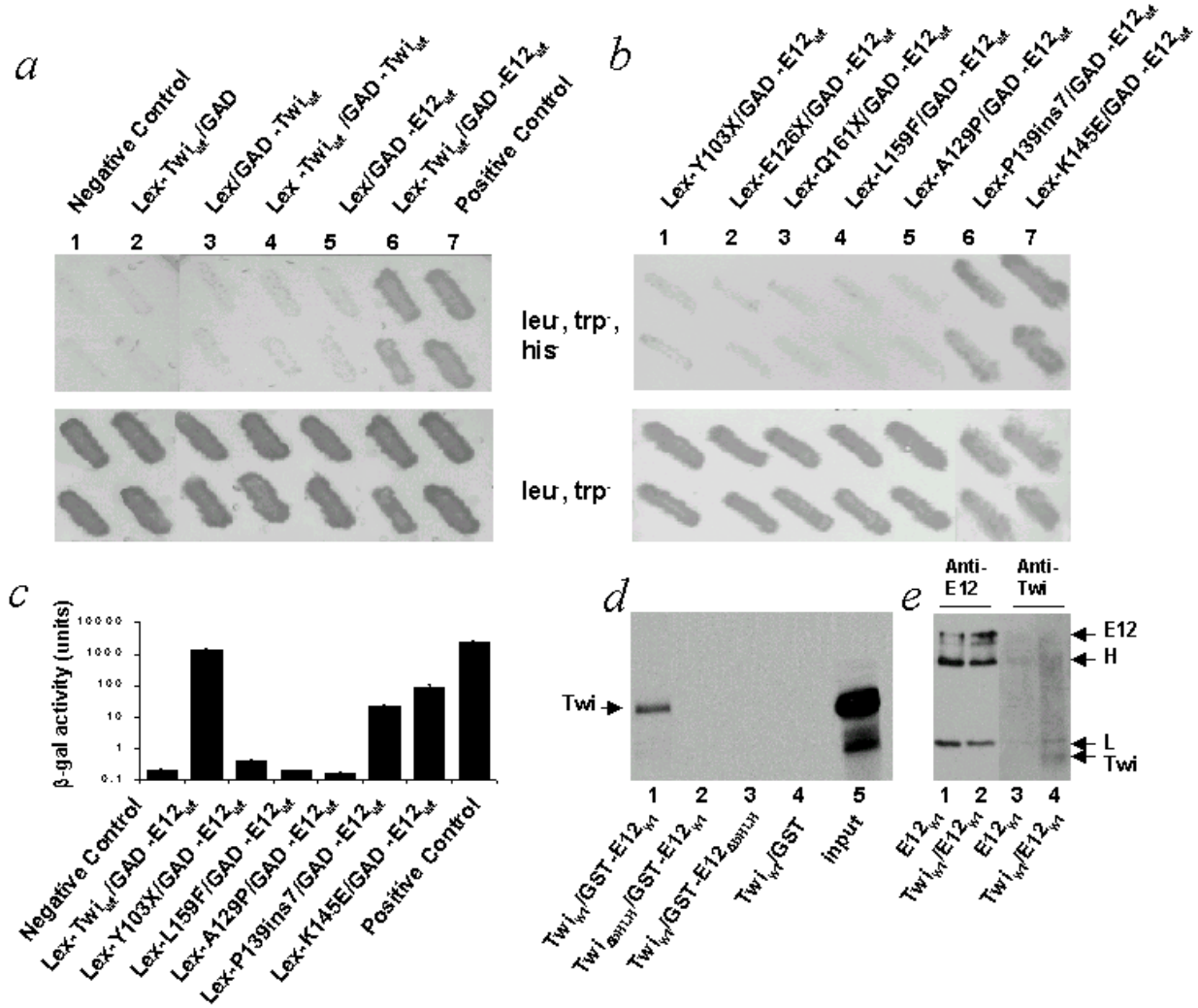
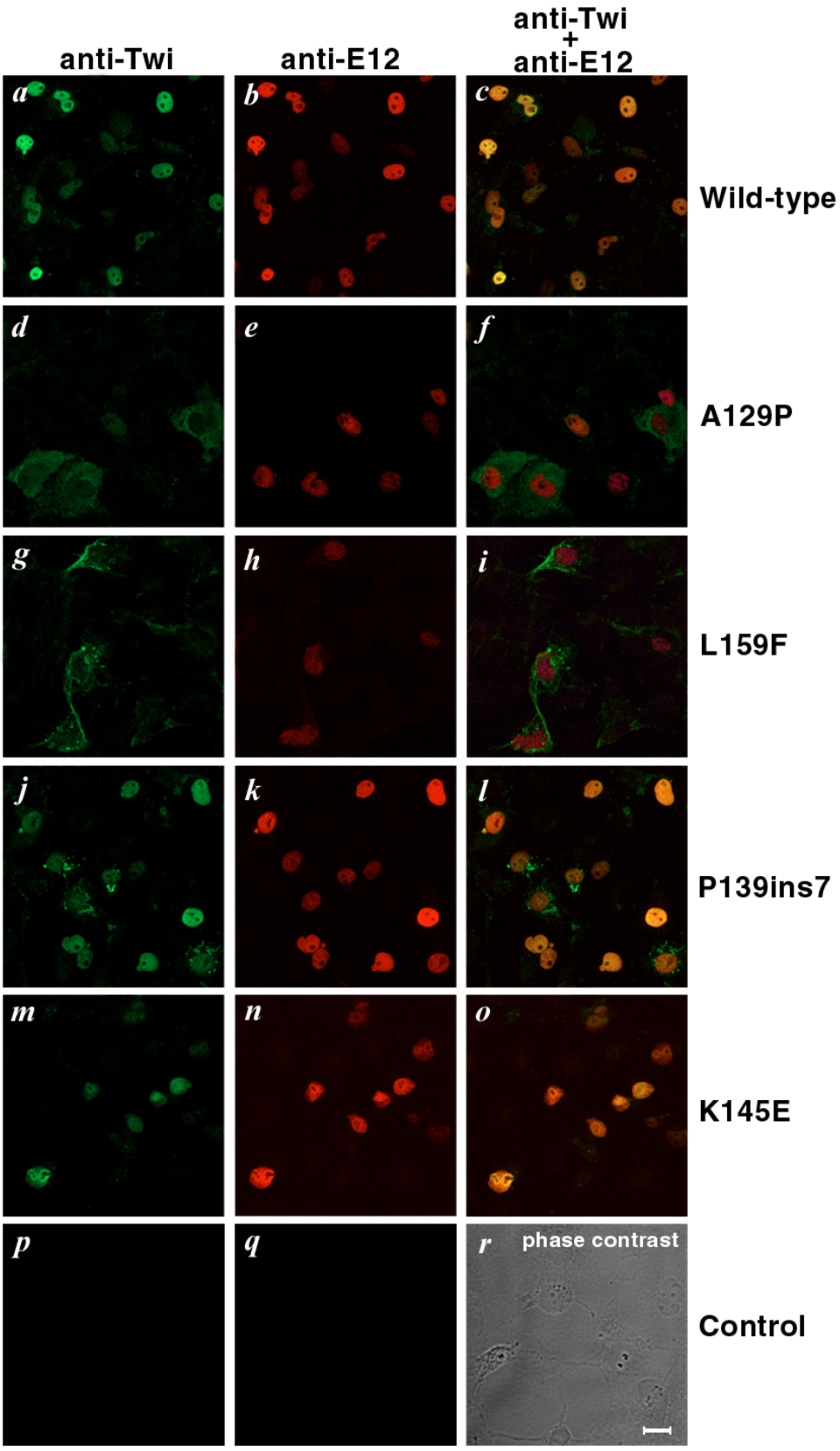
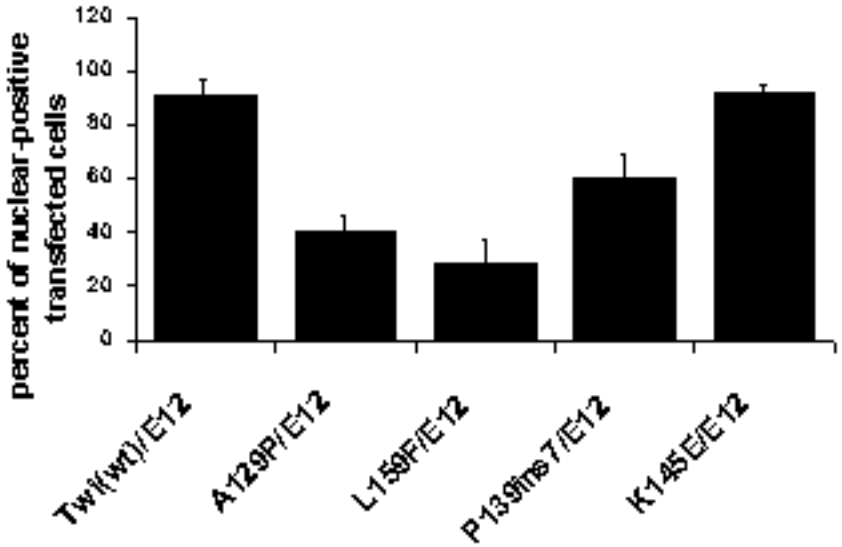
E126X
Q161X
A129P
L159F
K145E
P139ins7
1 2 3 4 5 6 7 8
48h

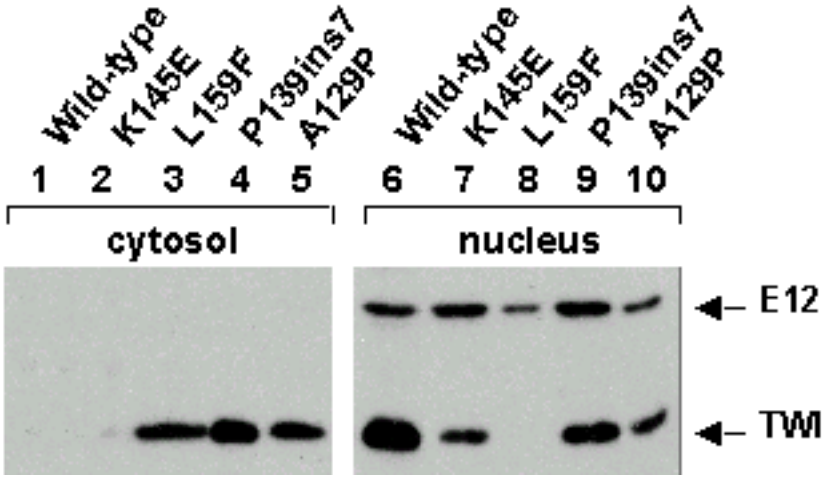

a
Negative Control
Lex-Twi$_{wt}$/GAD
Lex/GAD -Twi$_{wt}$
Lex -Twi$_{wt}$ /GAD -Twi$_{wt}$
Lex/GAD -E12$_{wt}$
Lex- Twi$_{wt}$/GAD -E12$_{wt}$
Positive Control
1 2 3 4 5 6 7
leu$^-$, trp$^-$, his$^-$
leu$^-$, trp$^-$
b
Lex-Y103X/GAD -E12$_{wt}$
Lex-E126X/GAD -E12$_{wt}$
Lex-Q161X/GAD -E12$_{wt}$
Lex-L159F/GAD -E12$_{wt}$
Lex-A129P/GAD -E12$_{wt}$
Lex-P139ins7/GAD -E12$_{wt}$
Lex-K145E/GAD -E12$_{wt}$
1 2 3 4 5 6 7
c
β-gal activity (units)
10000
1000
100
10
1
0.1
Negative Control
Lex- Twi$_{wt}$/GAD -E12$_{wt}$
Lex-Y103X/GAD -E12$_{wt}$
Lex-L159F/GAD -E12$_{wt}$
Lex-A129P/GAD -E12$_{wt}$
Lex-P139ins7/GAD -E12$_{wt}$
Lex-K145E/GAD -E12$_{wt}$
Positive Control
d
Twi
1 2 3 4 5
Twi$_{wt}$/GST-E12$_{wt}$
Twi $_{\Delta aa126}$/GST-E12$_{wt}$
Twi$_{wt}$/GST-E12$_{\Delta aa126}$
Twi$_{wt}$/GST
input
e
Anti-E12
Anti-Twi
E12
H
L
Twi
1 2 3 4
E12$_{wt}$
Twi$_{wt}$/E12$_{wt}$
E12$_{wt}$
Twi$_{wt}$/E12$_{wt}$


anti-Twi
anti-E12
anti-Twi
+
anti-E12
a
b
c
Wild-type
d
e
f
A129P
g
h
i
L159F
j
k
l
P139ins7
m
n
o
K145E
p
q
r
phase contrast
Control

*a*


percent of nuclear-positive transfected cells
120
100
80
60
40
20
0
Twi(wt)/E12
A129P/E12
L159F/E12
P139ins7/E12
K145E/E12

*b*


Wild-type
K145E
L159F
P139ins7
A129P
Wild-type
K145E
L159F
P139ins7
A129P
1 2 3 4 5
6 7 8 9 10
cytosol
nucleus
E12
TWI